Effects of condensed tannins in conifer leaves on the composition and activity of the soil microbial community in a tropical montane forest

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Abstract

Background and Aims Condensed tannins, a dominant class of plant secondary metabolites, play potentially important roles in plant-soil feedbacks by influencing the soil microbial community. Effects of condensed tannins on the soil microbial community and activity were examined by a short-term tannin-addition experiment under field and laboratory conditions.

Methods Condensed tannins were extracted from the leaves of a dominant conifer (*Dacrydium gracilis*) in a tropical montane forest on Mt. Kinabalu, Borneo. The extracted tannins were added to soils beneath the conifer and a dominant broadleaf *(Lithocarpus clementianus)* to evaluate the dependence of the response to tannin addition on the initial composition of the soil microbial community.

Results Enzyme activities in the field tannin-addition treatment were lower than in the deionized-water treatment. Carbon and nitrogen mineralization were also inhibited by tannin-addition. The fungi-to-bacteria ratio after tannin-addition was higher compared with the distilled-water treatment in the laboratory experiment.

Conclusions Based on our results, we suggest that the higher concentration of condensed tannins in the leaf tissues of *Dacrydium* than in those of *Lithocarpus* is a factor influencing the microbial community and activity. This may have influences on subsequent plant performance, which induces plant-soil feedback processes that can control dynamics of the tropical montane forest ecosystem.

40 **Key-words**

41 Condensed tannins; Conifer; Plant-soil feedback; Soil enzyme activity; Soil microbial

42 community; Tropical montane forest

43

Introduction

Condensed tannins, a dominant class of plant secondary metabolites, are often found in plant tissues in great abundance (Hartley and Jones 1997). For example, tree leaves often contain 10–20% of condensed tannins by dry weight (Kuiters 1990; Ushio and Adams 2011). Due to their specific biochemical properties, these metabolites have a large effect on soil mineralization processes, through their influence on the quality of organic matter, soil enzyme activity, and soil microbial community (Kraus et al. 2003; Smolander et al. 2012). For example, condensed tannins in soil can form recalcitrant complexes with proteins due to the presence of many hydroxyl functional groups in their molecular structure (Kraus et al. 2003). Further, soil microbial activity can be directly affected by the toxicity of condensed tannins or by the complexation and inactivation of the extracellular enzymes driving mineralization processes (Scalbert 1991). In practice, studies have indicated that condensed tannins often reduce the rates of carbon and/or nitrogen mineralization (Kraal et al. 2009; Schimel et al. 1998; Fierer et al. 2001; Kanerva et al. 2006).

Evidence suggests that condensed tannins in the leaf tissues of plants have a large effect not only on soil mineralization processes but also on subsequent plant nutrient acquisition and nutrient cycling in an ecosystem (Wurzburger and Hendrick 2009; Ushio et al. 2009; Northup et al. 1995). Therefore, condensed tannins have been recently highlighted as key substances in regulating the feedbacks between plants and soil. Moreover, studies have also suggested that plant–soil feedbacks are a potential driver of plant community assemblage and ecosystem processes (Wardle et al. 2004), such as

plant species coexistence (Frelich et al. 1993; Miki et al. 2010), nutrient cycling (Hobbie 1992), and plant community succession (Kardol et al. 2007; van der Putten et al. 2009). Therefore, an understanding of the influence of tannins on soil mineralization processes can provide insights into tannin regulation mechanisms of the ecosystem processes through their influence on plant-soil feedbacks.

The control of tannins on soil processes has been previously studied (Bowman et al. 2004; Fierer et al. 2001; Meier and Bowman 2008; Schimel et al. 1998; Kanerva et al. 2006; Kraal et al. 2009; Nierop et al. 2006). For example, Fierer et al. (2001) extracted four fractions (i.e., four molecular weight categories) of poplar (*Populus balsamifera*) condensed tannins and examined the effects of the tannin fractions on soil nitrogen cycling by incubation experiments. The addition of tannins reduced the nitrogen availability, although the exact mechanism differed depending on the molecular weight of the added tannins. Low-molecular-weight tannins generally served as a labile carbon source and stimulated microbial immobilization of nitrogen, whereas high-molecular-weight tannins appeared to inhibit degrading enzymatic activities by forming a recalcitrant complex with both the enzymes and their substrates. In addition, as suggested by Kanerva et al. (2006) and Kanerva and Smolander (2008), the low-molecular weight fractions probably contained compounds other than tannins such as waxes, chlorophyll and terpenoids, which can also contributes to the differences in soil responses to tannin addition between fractions. Although their experiments under laboratory conditions provide insights into the role of condensed tannins in nutrient cycling (Bowman et al. 2004; Fierer et al. 2001; Kanerva et al. 2006), the detailed mechanism of tannin-based regulation of soil mineralization processes is not fully

understood. This limitation is attributable to the lack of studies exploring the effect of tannins on the soil microbial community despite the finding that the composition and abundance of the soil microbial community fundamentally drives soil mineralization processes (Strickland et al. 2009; Balser and Firestone 2005).

The effects of tannins depend on differences in microbial abilities to resist tannin toxicity and the ability to utilize condensed tannins (Kraus et al. 2003; Scalbert 1991). Some groups of microbes (e.g., a group of saprophytic fungi) can utilize and grow on media containing a high concentration of condensed tannins. Furthermore, for fungi, the minimum inhibitory concentration of tannins is often higher than 0.5 g l^{-1} in a medium, but the value is generally lower in bacteria (Scalbert 1991 and references therein). Thus, a specific concentration of condensed tannins can result in selection for a specific group of microbes, altering the composition of the soil microbial communities. Further, the effects of condensed tannins on soil processes under field conditions with many influential factors have not been investigated, affecting our understanding of the mechanism by which tannins influence the soil microbial communities and soil processes.

A tropical montane forest on Mt. Kinabalu, Borneo, Malaysia, provides an opportunity to test the potential effects of condensed tannins on the soil microbial community and mineralization processes. The variations in leaf chemistry (e.g., the concentration of condensed tannins) of locally coexisting plants in the forest are likely to induce distinct spatial patchiness of the soil physicochemical and microbial properties. Indeed, our previous studies found that the composition and activities of the soil microbial community were different beneath different tree species, and the difference

was distinct especially between a dominant conifer, *Dacrydium gracilis* (Podocarpaceae) and a dominant broadleaf, *Lithocarpus clementianus* (Fagaceae) (Ushio et al. 2008; Ushio et al. 2010b). Specifically, saprophytic fungi were more dominant beneath the conifer species than beneath the broadleaf species. The spatial pattern of microbial properties corresponds well to the concentration gradient of condensed tannins in soil (e.g. fungi-to-bacteria ratio positively corresponds to the concentration of condensed tannins in soil; Ushio et al. 2010b), suggesting that condensed tannins are one of the factors responsible for these tree-specific spatial patterns. Moreover, as the nitrogen mineralization rate is constantly low in this forest (Kitayama et al. 2004a; Kitayama et al. 1998; Hall et al. 2004), it is also likely that the effect of tannins on the nitrogen mineralization rate influences subsequent plant nutrient acquisition.

In the tropical montane forest, tannin-addition experiments were conducted under both field and laboratory conditions to evaluate the effects of condensed tannins on soil mineralization processes and microbial communities. Our specific hypotheses are as follows: 1) the addition of condensed tannins will inhibit general microbial activities (i.e., soil enzyme activity, respiration rate, and nitrogen mineralization rate) by their protein-precipitation capacity; 2) the addition of condensed tannins will result in an increase in fungal dominance because of their utilization advantage for condensed tannins compared with the bacterial community; and 3) under *Dacrydium* the initial dominance of fungi will result in a lower effect of tannin addition due to greater fungal ability to degrade the added tannins.

Materials and methods

Site description and selected tree species

Soils for the experiment were collected from a permanent plot in a primary montane forest on the south slope of Mt. Kinabalu in Sabah, Borneo, Malaysia (summit height = 4095 m; 6°05′N, 116°33′E); the tannin-addition experiment was conducted in this plot and also under controlled laboratory conditions. The study was conducted from January to March 2008. The research plot is located at 1560 m above sea level, near the headquarters of the Kinabalu Park. The climate is humid and tropical with a mean annual air temperature of 18°C and annual precipitation of 2714 mm (Aiba and Kitayama 1999). This area does not have marked seasonality but shows slight variation in the monthly precipitation. The plot is covered with evergreen broadleaf trees interspersed with conifers (relative basal area of conifers ~ 15%), including 109 tree species per hectare with diameter >10 cm at breast height (Aiba et al. 2002). The decomposition rate of standing litter is low compared with that at lowland sites because of the relatively low temperature. Thus organic soil horizon in this plot is thick (ca. 5 cm), and the soil is acidic (pH measured in water is ~ 4.0). The plot is in the last stage of pedogenesis, and the soil contains a low concentration of phosphorus, which is thought to limit plant growth (Kitayama et al. 2004a). Moreover, the measurement of net nitrogen mineralization rate in topsoils (0-15 cm depth) of this forest revealed that the mineralization rate was nearly zero, or often negative over a year, indicating that plant performance can also be limited by the nitrogen availability (Hall et al. 2004; Kitayama

158 et al. 2004a; Kitayama et al. 1998). In this forested area, two dominant tree species were
159 selected: *Dacrydium gracilis* (Podocarpaceae; conifer) and *Lithocarpus clementianus*
160 (Fagaceae; broadleaf tree). This is because the leaf chemistry of these species is
161 markedly different, especially with regard to the concentration of condensed tannins
162 (*Dacrydium*, 6.38%; *Lithocarpus*, 0%; Suzuki, S. unpublished data), and because the soil
163 microbial and physicochemical properties are also different beneath the two species
164 (Table S1, Ushio et al. 2008; Ushio et al. 2010b).
165
166 Tannin extraction
167
168 Condensed tannins were prepared as described by Fierer et al. (2001) with the following
169 modifications. Fresh leaves of *Dacrydium* were collected from tree individuals growing
170 in this forested area, and the leaves were immediately taken back to the laboratory. The
171 leaves were freeze-dried (yielded ~150 g of dried leaves), finely ground and rinsed three
172 times with n-hexane, following which n-hexane was discarded. The remaining leaf
173 material was extracted three times with 70% acetone, and the three extracts were
174 combined and concentrated by evaporation with a vacuum pump (DIVAC 0.6 L; Tokyo
175 Rikakikai Co. Ltd., Tokyo, Japan). The concentrated extract was fractionated six times
176 with 100% ethyl acetate, after which the ethyl acetate (hydrophobic) fraction containing
177 low-molecular-weight phenolics was discarded. This is because the fraction appeared to
178 influence the soil processes in a very different manner when compared with
179 high-molecular-weight phenolics such as condensed tannins, and because this fraction
180 contains a minor fraction of tannins (Fierer et al. 2001; Kanerva et al. 2006). The

remaining 70% acetone fraction was concentrated by freeze-drying and loaded onto a Sephadex LH-20 (GE Healthcare, UK, Ltd., England) chromatography column (gel length = 32 cm, flow rate ≈ 1.5 ml min^{-1}). The column was eluted with 50% methanol followed by 70% acetone. The acetone fraction was then collected and concentrated by evaporation followed by freeze-drying. This fraction was thought to contain high-molecular-weight tannins (Fierer et al. 2001; Kanerva et al. 2006). Extraction of approximately 150 g *Dacrydium* leaves yielded 8.5 g of condensed tannins (5.46% of the initial dry weight). This value is reasonable in comparison with the quantification value of condensed tannins (6.38%; Suzuki S. unpublished data) with the acid-butanol method (Porter et al. 1986).

Tannin-addition experiment under field conditions

Five replicate individuals of the *Dacrydium* and *Lithocarpus* were selected in the forest plot. Two soil collars (diameter × height; 10 cm × 10 cm) were buried beneath each tree crown of the selected individuals for two weeks before the tannin-addition experiment. One collar was for tannin addition-treatment, and the other one was for control (deionized water-addition treatment). Most fine roots inside these collars were separated from plant individuals by soil collar insertion, but possible effects of deeper roots could not be excluded in this study. Organic matter inputs from the excised roots may hamper effects of condensed tannins on soil processes, but the organic matter, especially for labile fractions, can be consumed during the two-weeks stabilization. After the two-weeks stabilization, 350 mg of condensed tannins per soil collar in deionized water

was added for the tannin treatment, resulting in approximately 20- to 30-fold higher than the concentration of condensed tannins in intact soil (Ushio et al. 2010b). Only the soil respiration rate was monitored one week after the addition of condensed tannins to avoid destructive soil sampling for the other analyses. The soil samples inside the soil collars were collected after one week of monitoring, and their pH, enzyme activity, and microbial community (lipid profile) were analyzed.

Laboratory incubation experiments

Five replicates of the two tree species were selected, and soil samples for the laboratory incubation experiment were collected from the surface organic soil layer (*ca.* 0-5 cm depth) beneath these individuals. Before applying them to the incubation experiments, the soils were maintained at 20°C in the dark with a constant moisture condition for a week to stabilize the soil conditions. Each soil sample was divided for two treatments: control (i.e., deionized water-addition treatment) and condensed tannin-addition treatment. Approximately 60 g of each fresh soil sample was used for this experiment.

Extracted high-molecular-weight condensed tannins were added in soluble form in deionized water to the five soil samples of both tree species at the equivalent of 10 mg condensed tannins per gram dry soil, resulting in concentrations of approximately equal to 20- to 30-fold higher than the concentration of condensed tannins in intact soil. After the addition, a subset of each replicate soil sample was sampled along the time course of 1, 3, 7, 14, and 21 days. Therefore, 100 soil samples (two tree species × two treatments × five replicates × five sampling days) were obtained totally.

227

228 Soil physicochemical and microbial properties

229

230 The soil pH, respiration rate, enzyme activity, and microbial community (lipid profile) in
231 each sample were analyzed. The properties of the intact soil samples (i.e., before the
232 samples were divided for the treatments) were also analyzed (see Table S1). The soil pH
233 in water and 0.01N KCl was measured with soil-to-solution ratios of 1:5 and 1:10,
234 respectively. The soil respiration rate was measured with a photosynthesis instrument
235 (LI-6400; LI-COR Biosciences, Lincoln, NE, USA) fitted with a closed soil respiration
236 chamber, which was then directly fitted to a half-buried soil collar to measure the
237 respiration rate under field conditions. In the case of the laboratory incubation
238 experiment, incubated soil was placed in a column-shaped box, and its respiration rate
239 was directly measured with the same system. Another set of soil samples (i.e., two tree
240 species × two treatment ×five replicates) was prepared for nitrogen mineralization
241 experiment. Net nitrogen mineralization rates were determined by comparing the initial
242 concentration of inorganic nitrogen (i.e., ammonium + nitrate) with the final
243 concentration of inorganic nitrogen after two weeks of incubation at 20°C in the dark
244 with a constant moisture condition. The concentration of inorganic nitrogen was
245 measured colourimetrically (FUTURA; Alliance).

246 For the soil enzyme analyses, the potential activities of acid phosphatase,
247 β-D-glucosidase, *N*-acetylglucosaminidase, phenol oxidase, and peroxidase were
248 measured. The soil samples were maintained in a refrigerator at 4°C for up to three days
249 before the enzyme activities were measured. For acid phosphatase, β-D-glucosidase, and

N-acetylglucosaminidase analyses the modified method of Tabatabai and Bremer (1969) was used. A standard curve was developed using *p*-nitrophenol. For phenol oxidase and peroxidase, 3,4-dihydroxy-L-phenylalanine (DOPA) was used as substrate, and a standard curve was developed using DOPA and tryrosinase. The detailed method was described in our previous studies (Ushio et al. 2010a; Ushio et al. 2010b).

For the analysis of microbial lipid biomarkers (White and Ringelberg 1998), each of soil samples was well mixed, frozen, and then freeze-dried immediately after soil sampling. We extracted, purified and identified PLFAs from microbial cell membranes using a hybrid lipid extraction based on a modified Bligh and Dyer (1959) technique, combined with fatty acid methyl ester analysis (FAME) as described by Microbial ID Inc. (Hayward, CA). The detailed method was described in Ushio et al (2010b). Total lipid abundance was calculated as a sum of lipids of which chain length was from 10 to 20. Indicator lipids used for calculation were as follows. Total bacterial biomass was estimated by the sum of the abundance of *i*14:0, 15:0, *i*15:0, *a*15:0, *i*16:0, 17:0, *i*17:0, *a*17:0, 16:1ω7, *cy*17:0 and *cy*19:0 (Frostegård et al. 1993; Mentzer et al. 2006). Gram-positive bacteria were represented by the branched lipids including *i*14:0, *i*15:0, *a*15:0, *i*16:0, *i*17:0, and *a*17:0 (Zelles et al. 1995), whereas Gram-negative bacteria were represented by the mono-saturated and cyclopropyl lipids including 16:1ω7, *cy*17:0 and *cy*19:0 (Ratledge and Wilkinson 1988; Yao et al. 2000). Fungal biomass was estimated by the abundance of 18:2ω6,9 (Frostegård et al. 1993). Fungi-to-bacteria ratio and Gram-positive bacteria to Gram-negative ratio were calculated as 18:2ω6,9/(sum of all bacterial lipid), and (sum of the branched lipids)/(sum of the mono-unsaturated and cyclopropyl lipids), respectively.

Statistics

Effects of condensed tannins, tree species and incubation time in the laboratory incubation experiment were tested by additive mixed models (Wood 2004) since it was likely that the effects of incubation time on the soil properties were not linear (smoothing function was applied for the effect of incubation time) and because we did not have *a priori* hypothesis about the relationship between incubation time and the soil properties. Model formulation for the field experiment was as follows: explained variable = tree species + treatment + treatment × tree species + random effect of each soil sample. Model formulation for the laboratory incubation experiment was as follows: explained variable = tree species + treatment + treatment × tree species + days after tannin addition + random effects of each soil sample. Effects of condensed tannins and tree species in the field experiment were tested by linear mixed models. Statistical analyses for the field tannin-addition experiment were conducted only on the data obtained after tannin addition. Principal component analysis (PCA) was performed for the lipid data to evaluate the overall composition of the microbial communities. Before conducting this analysis, the values of the concentrations of indicative lipids were divided by the total lipid abundance for evaluating the relative abundance of each lipid biomarker. To ensure normality in the lipid data set, the values were transformed by considering the negative arcsine of the square root of mole percent (Balser and Firestone 2005). The ‘mgcv’ (Wood 2004) and ‘nlme’ (Pinheiro et al. 2009) packages were used for the additive mixed model analysis and linear mixed model analysis, respectively. All

296 statistical analyses were conducted by using the free statistical environment R (R
297 Development Core Team 2011). In the analyses, $P < 0.05$ was regarded as significant. In
298 addition, $0.05 < P < 0.1$ was regarded as marginally significant, considering the inherent
299 spatial heterogeneity of the soil properties in surface organic layer.
300

Results

Intact soil properties

Properties of intact soils differed beneath each tree species, *Dacrydium* and *Lithocarpus* (Table S1). A higher concentration of condensed tannins and a higher fungi-to-bacteria ratio were measured in the soils beneath the *Dacrydium* than in those beneath the *Lithocarpus*. Lower water-extracted soil pH, lower activity of *N*-acetylglucosaminidase, lower nitrogen mineralization rate, and lower rate of soil respiration were detected in the soils beneath the *Dacrydium*. On the other hand, no significant differences were observed in the other soil properties. These trends were generally consistent with those of the spatial pattern of soil physicochemical and microbial properties underneath these tree species (Ushio et al. 2008; Ushio et al. 2010b).

Tannin-addition experiments under field conditions

The activities of acid phosphatase, β-D-glucosidase, and *N*-acetylglucosaminidase were significantly influenced by the tannin addition (Table 1). Mean values of the three enzyme activities were lower after the tannin-addition treatment than the control treatment, and the effects were similar in the cases of both the *Dacrydium* and the *Lithocarpus* soil samples (Fig. 1a–c). A marginally significant effect of the interaction between tree and tannins was found on the phenol oxidase activity: the activity after tannin addition was lower than that after the control treatment in the *Dacrydium* soil

samples, but this was not the case for the *Lithocarpus* soil samples (Fig. 1d). There were no significant effects of the interaction of tree × tannins on the soil enzyme activities and respiration rate except for phenol oxidase, indicating that condensed tannins had similar effects on these parameters (except for the phenol oxidase activity) in both the *Dacrydium* and the *Lithocarpus* soil samples. In general, tannin addition also had a significant inhibition effect on the soil respiration rate (Table 1). Specifically, the respiration rate was lower from 3.5 h to 7 d in the tannin-treated soils (Fig. 2). A significant tree × tannin interaction effect was found on the water-extracted soil pH (Table 1).

The addition of condensed tannins did not significantly influence total lipid abundance, fungi-to-bacteria ratio, or gram-positive bacteria to gram-negative bacteria ratio (Table 1). PCA revealed the response of the overall composition of the microbial community to the addition of condensed tannins. Here, only the first three principal components (PCs) are reported: PC1, PC2, and PC3 explained 40.6%, 18.2%, and 10.5% of the total variation, respectively. The addition of condensed tannins did not significantly influence any of the PCs (Table 1). Weak effects of the interaction between tree and tannins on the gram-positive bacteria to gram-negative bacteria ratio were observed. Tree species had significant and strong effects on the fungi-to-bacteria ratio and a part of the overall microbial composition (Table 1), which is consistent with the results of the previous studies (Ushio et al. 2008; Ushio et al. 2010b).

Laboratory incubation experiment

The addition of condensed tannins significantly influenced the activity of phenol oxidase (Table 2): the activity after the tannin-addition treatment was significantly lower than that after the control treatment, and this trend was true for both *Dacrydium* and *Lithocarpus* soil samples (Figs. 3d and 4d). No significant effects of tannin addition on other soil enzyme activities were detected. A significant effect of the interaction of tree × treatment was found on the activity of peroxidase (Table 2). The activity of peroxidase after the tannin-addition treatment was higher than after the control treatment in the case of the *Lithocoarpus* soil samples during 1–14 d, but was almost the same in the case of the *Dacrydium* soil samples irrespective of the treatment (Figs. 3e and 4e). Soil respiration rate was not influenced by the addition of condensed tannins (Table 2). However, soil respiration rate after the tannin-addition treatment was slightly lower than those after the control treatment for 3–21 d (Figs. 3f and 4f), which resulted in a marginally significant effect of tannin-addition on cumulative carbon dioxide emission (Table 2 and Fig. S1). Tree × treatment interaction effects were found in the case of cumulative carbon dioxide.

The fungi-to-bacteria ratio was significantly higher after the tannin-addition treatment than the control treatment (Table 2 and Fig. 5). The mean concentration of the primary fungal biomarker lipid (18:2ω6,9) was slightly higher after the tannin-addition treatment, whereas the mean concentration of the sum of bacterial lipids was lower after the addition of condensed tannins though the differences were not significant (data not shown). No significant change was found in the total lipid abundance by tannin addition (Table 2). Further, the gram-positive bacteria to gram-negative bacteria ratio was marginally significantly higher after the tannin-addition treatment (Table 2). PCA

370 revealed overall microbial community compositional response to the addition of
371 condensed tannins. Here, only the first three PCs are reported. PC1, PC2, and PC3
372 explained 39.8%, 21.8%, and 9.5% of the total variation, respectively. The addition of
373 condensed tannins influenced PC2 significantly, but PC1 and PC3 were not influenced
374 (Table 2). No significant effects of the interaction between tree and treatment were
375 observed on soil microbial properties.

376 The net nitrogen mineralization rate was significantly lower in the soils beneath
377 the *Dacrydium* (Fig. 6). The addition of condensed tannins decreased net nitrogen
378 mineralization rate in the *Lithocarpus* soil samples, but not significantly in the
379 *Dacrydium* soil samples. The KCl-extracted soil pH was slightly but significantly lower
380 after the tannin-addition treatment than the control treatment (Table 2).
381

Discussion

Effects of tannin-addition on soil microbial activities

The first hypothesis, the addition of condensed tannins will inhibit microbial activities, was generally supported by the field and laboratory experiments. We found that tannin addition significantly lowered the activities of acid phosphatase, β-D-glucosidase and *N*-acetylglucosaminidase in the field experiment as well as phenol oxidase activity in the laboratory incubation experiment (Table 1 and 2). Condensed tannins have the ability to form a recalcitrant protein–tannin complex (Kraus et al. 2003), which can inhibit soil enzyme activity; thus, the lower enzyme activities could be due to precipitation of the enzymes with tannins. The soil respiration rate after tannin treatment was significantly lower from 3.5 h to 7 d in the field experiments (Fig. 2). In addition, cumulative carbon dioxide emission was significantly lower after tannin treatment in the laboratory experiment (Table 2 and Fig. S1). These results are in agreement with the previous studies (Fierer et al. 2001; Schimel et al. 1998). Reduction of soil respiration could be due to either the inhibitory effects of tannins on the carbon-degrading enzyme activity (i.e., β-D-glucosidase and phenol oxidase) or their direct toxicity to soil microbes (Kraus et al. 2003; Smolander et al. 2012; Scalbert 1991).

On the contrary to the above-mentioned enzymes , no significant inhibitory effects of condensed tannins on the other soil enzyme activities were found (Table 1 and 2). The inhibitory effects of condensed tannins on enzymes may differ depending on the chemical characteristics of the enzyme species (Kraus et al. 2003). In addition, it is

interesting that the inhibitory effect of tannin addition on the enzyme activities (i.e. acid phosphatase and β-D-glucosidase) found in the field experiment was not detected in the laboratory experiment. Under field conditions, external substrates are continuously supplied to soil from leaf litter and throughfall, which may contribute to the slightly higher activities of enzymes than under laboratory condition (Figs. 1, 3 and 4). The higher microbial activity in the control treatment may render the inhibiting effect of condensed tannins clearer under field conditions than under laboratory conditions.

Reduction of nitrogen mineralization rate by condensed tannins was observed in the *Lithocarpus* soils in this study (Fig. 6) as well as in previous studies (Fierer et al. 2001; Schimel et al. 1998). The tannin–protein complex is recalcitrant, and is generally difficult for soil microbes to degrade (Majuakim 2005; Kraus et al. 2003). Therefore, the mineralization pathway from protein to inorganic nitrogen is often inhibited. Precipitation can also inhibit the activity of nitrogen-degrading enzymes (Kraus et al. 2003, and references therein), and consequently inhibit nitrogen mineralization. However, the activity of *N*-acethylglucosaminidase (one of the nitrogen-degrading enzymes) was not significantly lower after tannin addition (Table 2). Therefore, the reduction of the nitrogen mineralization rate observed in the *Lithocarpus* soil samples appears to be primarily due to the formation of recalcitrant tannin–protein complex with their protein substrate. In addition, toxic effects of the tannins on soil microbes may contribute to the reduction of the nitrogen mineralization rate (Kraus et al. 2003). It is possible, however, that condensed tannins might have an inhibitory effect on the activities of nitrogen-degrading enzymes that were not examined in this study.

Unlike the effect of condensed tannins on nitrogen mineralization rate in

Lithocarpus soils, no significant decrease in the net nitrogen mineralization rate was found in the tannin-treated *Dacrydium* soil samples versus controls (Fig. 4). The net nitrogen mineralization rate in the *Dacrydium* soil samples may be lower due to the relatively high background concentration of condensed tannins, and thus, further addition of condensed tannins to these samples may not reduce the nitrogen mineralization rate. This result partly supports our third hypothesis that effects of tannins will be less significant under *Dacrydium* than under *Lithocarpus*. We note that the values of the net nitrogen mineralization rate were considerably higher than those reported in the studies previously conducted at this site (Kitayama et al. 1998; Kitayama et al. 2004a). One possible reason for this difference is the difference in the soil sampling depth (i.e., surface organic soils [*ca*. 0-5 cm depth] versus deeper mineral soils [0-15 cm depth]), sampling place (sampling places in the forest were not exactly the same among the studies) and/or methodological differences (e.g., laboratory incubation versus the buried bag method). Both soil depth and incubation methodology can significantly influence the values of inorganic nitrogen concentration (Piccolo et al. 1994; Neill et al. 1997).

Effects of tannin-addition on the composition of the soil microbial community

The second hypothesis, the addition of condensed tannins will result in an increase in fungal dominance, was also supported by the laboratory incubation experiments. Tannin addition had significant effects on the composition of the soil microbial community in the laboratory incubation experiment (Table 2). Specifically, the fungi-to-bacteria ratio

after tannin-addition was higher than that in the control treatment (Fig. 5), due to the higher concentration of a saprophytic fungal biomarker lipid and the slightly lower concentration of bacterial biomarker lipids after the tannin-addition treatment (data not shown). Although cautions are needed to interpret the lipid data (see Frostegârd et al. 2011), the concentration of a lipid biomarker is still an good estimate of microbial biomass (White et al. 1979; Frostegård et al. 1991). Thus we conclude that tannin addition may result in an increase in saprophytic fungal biomass in the laboratory experiment. The saprophytic fungal community can often utilize condensed tannins as their energy source more effectively than the bacterial community, and can be more tolerant to the toxicity of condensed tannins (Scalbert 1991). These properties of the fungal community may be advantageous in soils with the tannin addition, and may consequently result in a higher fungi-to-bacteria ratio. In addition, at least a portion of the overall composition of the microbial community can be altered by tannin addition, based on the significant tannin-effect on the PC2 (Table 2). No significant tree × tannin interaction effects on the properties of the soil microbial community (Table 2) suggested that condensed tannins influenced the composition of the microbial community in a similar manner in both the *Dacrydium* and the *Lithocarpus* soils under the laboratory condition.

Although tannin addition had a significant effect on the fungi-to-bacteria ratio in the laboratory experiment, no significant changes were found in this ratio in the field experiment (Table 1). Additionally, in terms of the overall composition of the microbial community, PCs were unchanged (Table 1). Therefore, tannins had very weak or no effects on the composition of the soil microbial community in the field experiment. One

reason for the different results between the laboratory experiment and the field experiment could be that a portion of the condensed tannins may have leached into deeper soil layers or was flushed by rainfall (rainfall event occurred three times during the course of the one-week tannin-addition experiment under field conditions), weakening their influence.

Implications for plant-soil feedbacks in the tropical montane forest

Based on our results, we suggest that a higher concentration of condensed tannins in leaf tissues of the *Dacrydium* than those of the *Lithocarpus* could be one of the factors inhibiting the carbon and nitrogen mineralization process in the soil beneath tree crowns of *Dacrydium*. Since nitrogen availability is thought to limit plant productivity in this weathered tropical montane forest (Kitayama et al. 2004b), condensed tannins can have significant influences on performance of plants (e.g. nitrogen acquisition and/or growth rate of plants) through their effects on nitrogen mineralization process, as already suggested by our theoretical study (Ushio et al. 2009). For example, plants growing on soil beneath *Dacrydium* might have lower growth rates than those beneath *Lithocaprus* because of the lower nitrogen availability. Also, the tannin-induced feedback effects might depend on plant nitrogen acquisition strategy such as mycorrhizal fungal association, but such studies are still lacking. Therefore, future studies should focus on the performance of the plants growing on the soil beneath the tree crowns of the two species as well as mycorrhizal fungal associations. Because the conifers (Podocarpaceae) are a unique component of tropical montane forests (Kitayama et al.

2011), and because plant-soil feedbacks are an important driver to regulate forest ecosystem dynamics (Wardle et al. 2004), such studies on the plant-soil feedbacks will improve our understanding of the dynamics of tropical montane forest ecosystems.

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501

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511

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653
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Figure captions

Fig. 1 Effects of condensed tannins on the soil enzyme activities under field conditions. The white and gray columns indicate the control and tannin-addition treatments, respectively. The initial soil enzyme activities (i.e., before water or tannin addition) are not shown in this figure. †$P < 0.1$ and *$P < 0.05$ by paired *t*-test. The bars indicate the SEM.

Fig. 2 Effects of condensed tannins on the soil respiration rate under field conditions. The results of the *Dacrydium* and *Lithocarpus* soil samples were combined because no significant effect of the tree species on the soil respiration rate was found. Therefore, only the effects of tannin addition are shown here. The white and gray columns indicate the control and tannin-addition treatments, respectively. *$P < 0.05$, **$P = 0.01$, by paired *t*-test. The bars indicate the SEM.

Fig. 3 Effects of the addition of condensed tannins and duration of incubation on the enzyme activities and respiration rate of the *Dacrydium* soil samples. The activities of acid phosphatase (a), β-D-glucosidase (b), *N*-acetylglucosaminidase (c), phenol oxidase (d), and peroxidase, (e) as well as the soil respiration rate (f) are shown. The white and gray symbols indicate the control and tannin-added samples, respectively. Solid lines and dotted lines indicate predicted values of control and tannin treatment, respectively, and they are shown only when effects of tannin-addition or the interaction term were significant. The values were predicted by the additive mixed model. The bars indicate the SEM.

Fig. 4 Effects of the addition of condensed tannins and duration of incubation on the enzyme activities and respiration rate of the *Lithocarpus* soil samples. The activities of

678 acid phosphatase (a), β-D-glucosidase (b), *N*-acetylglucosaminidase (c), phenol oxidase
679 (d), and peroxidase (e), as well as the soil respiration rate (f) are shown. The white and
680 gray symbols indicate the control and tannin-added samples, respectively. Solid lines
681 and dotted lines indicate predicted values of control and tannin treatment, respectively,
682 and they are shown only when effects of tannin-addition or the interaction term were
683 significant. The values were predicted by the additive mixed model. The bars indicate
684 the SEM.

685 **Fig. 5** Effects of tree species, condensed tannins and incubation time on
686 fungi-to-bacteria ratio. *Dacrydium* and *Lithocarpus* soil samples are represented by
687 circles and triangles, respectively. The control and tannin-added samples are represented
688 by white and gray symbols, respectively. Solid lines and dotted lines indicate predicted
689 values of control and tannin treatment, respectively. The bars indicate the SEM. The
690 values were predicted by the additive mixed model. The results of the statistical analyses
691 are shown in Table 1.

692 **Fig. 6** Effects of the addition of condensed tannins and tree species on the net
693 nitrogen mineralization rate. The white and gray columns indicate the control and
694 tannin-addition treatments, respectively. *, † indicate significance level at $P < 0.05$ by
695 paired *t*-test and *t*-test, respectively. Bars indicate the SEM.

696

697 **Supplementary Figure Captions**

698 **Fig. S1** Cumulative carbon dioxide respired from soil samples in the laboratory
699 experiment. The white and gray bars indicate the control and tannin-addition treatments.
700 The bars indicate the SEM. The results of the statistical analyses are shown in Table 2.

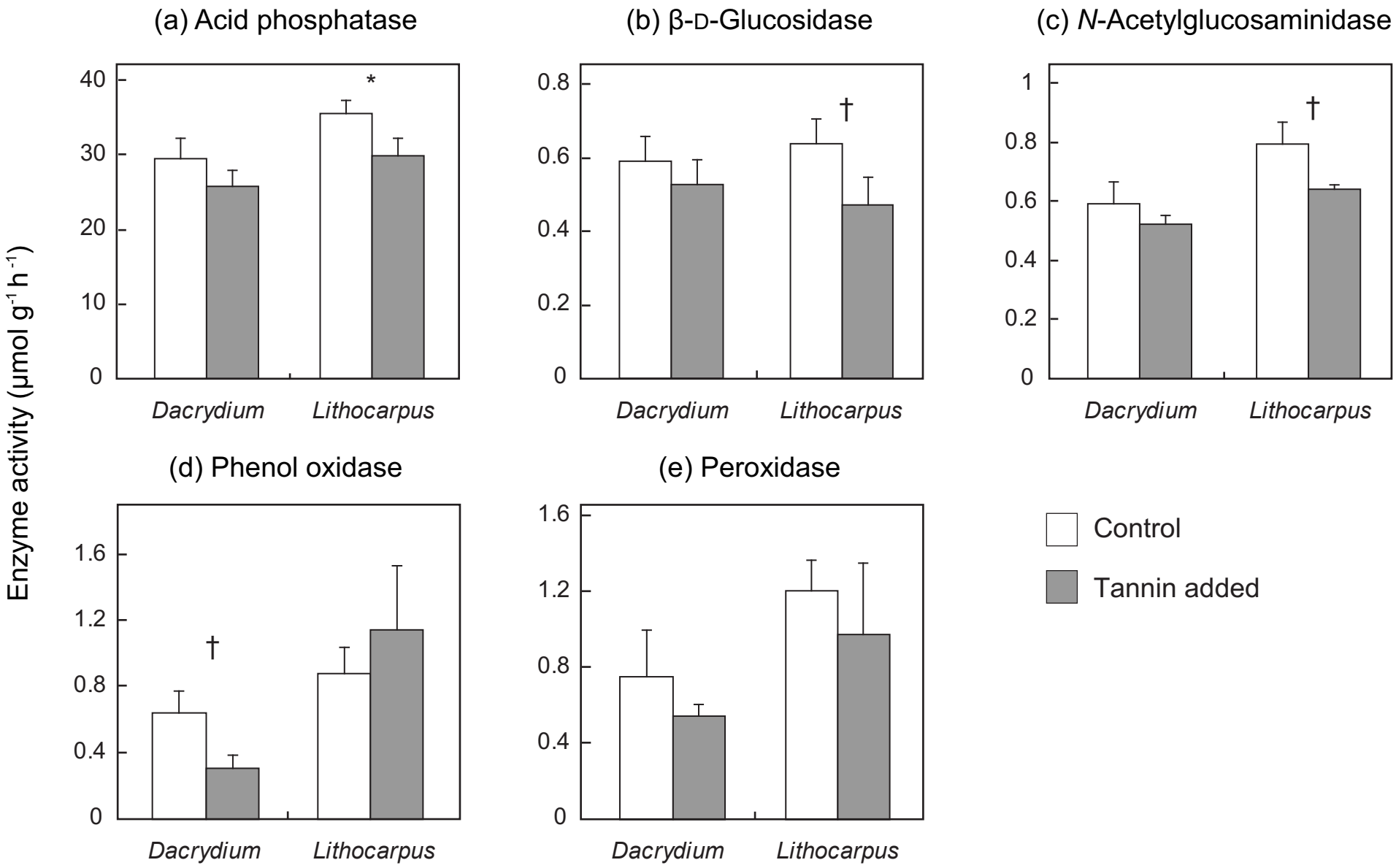

Fig. 1 Ushio et al.

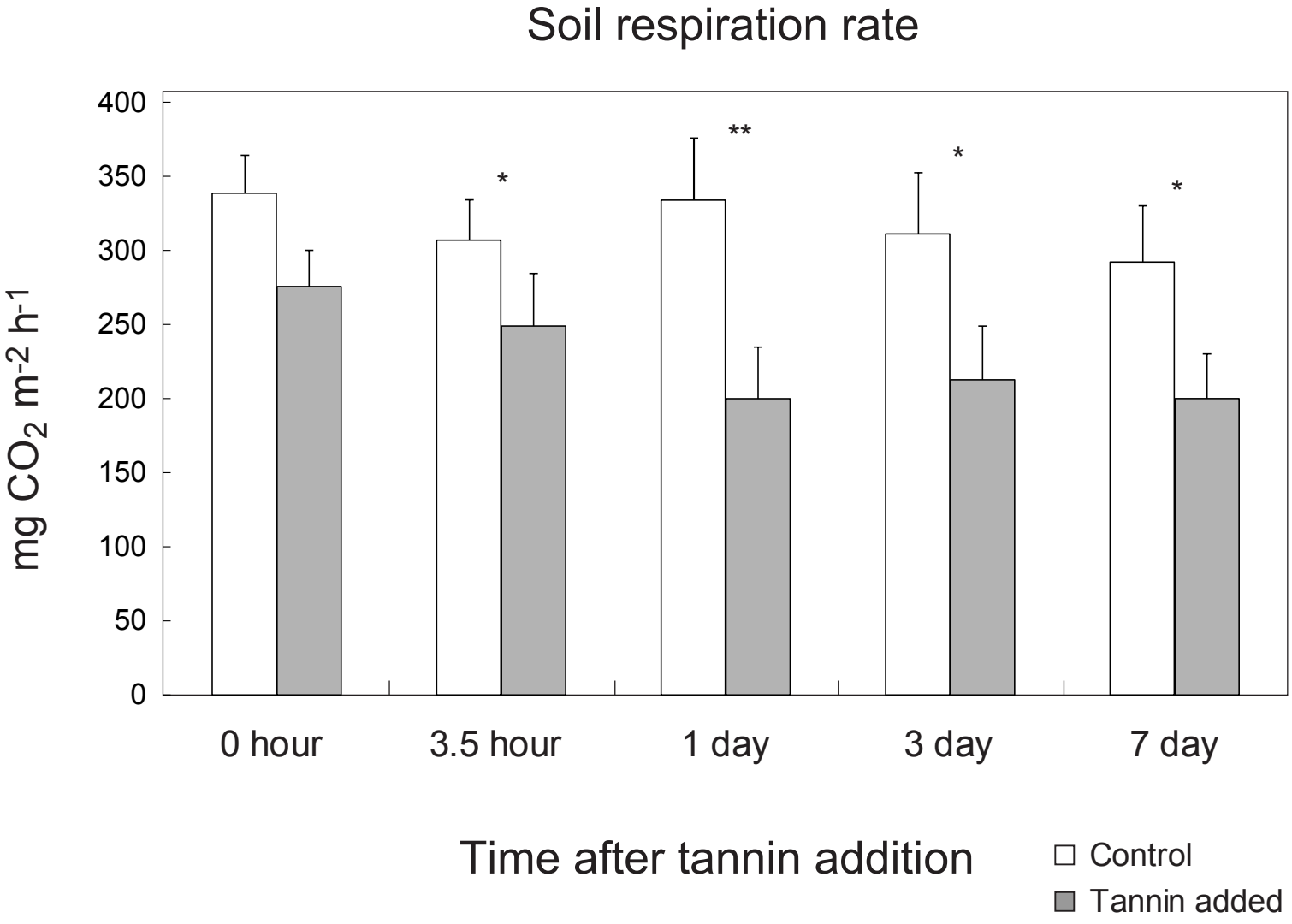

Fig. 2 Ushio et al.

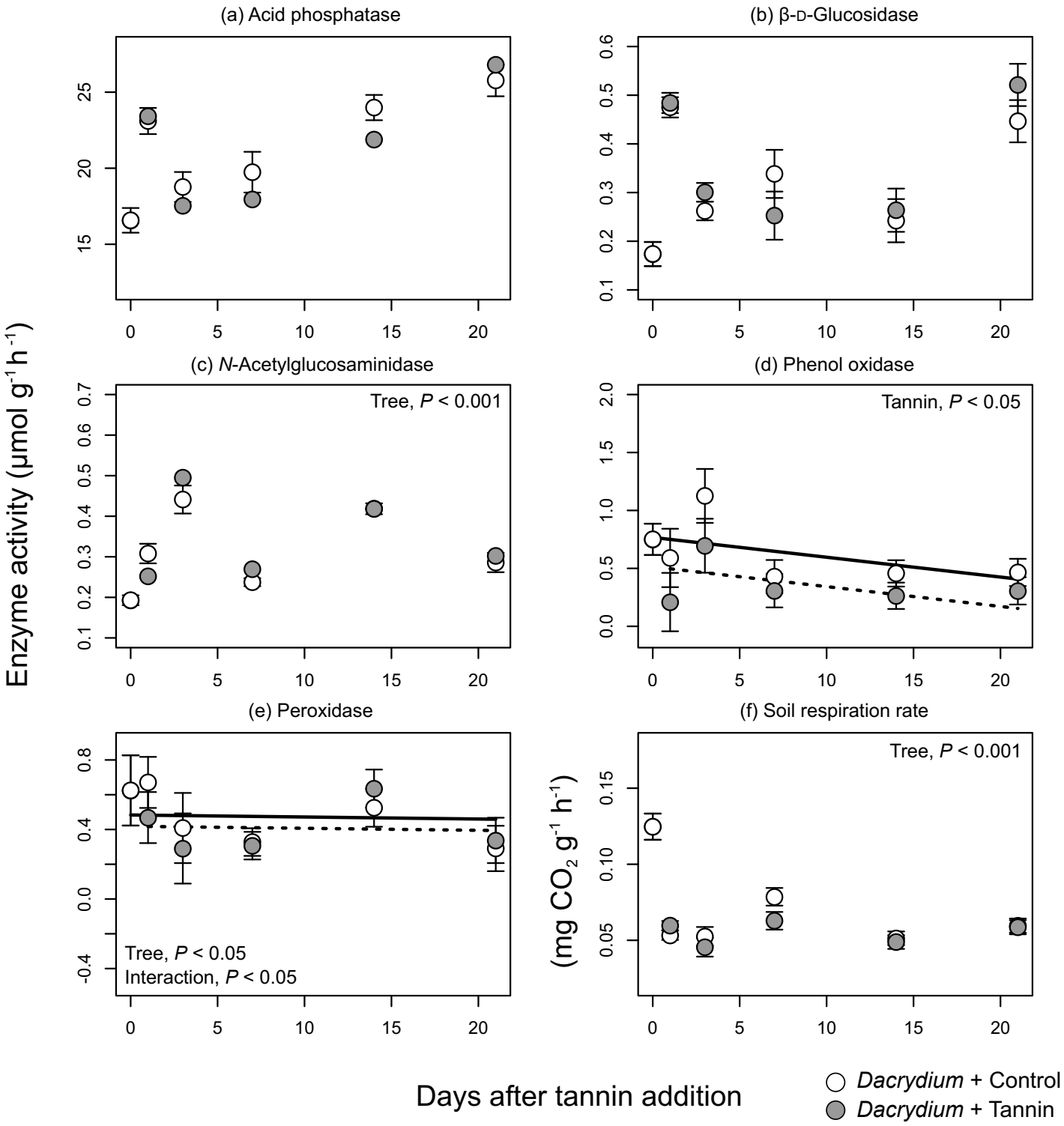

Fig. 3 Ushio et al.

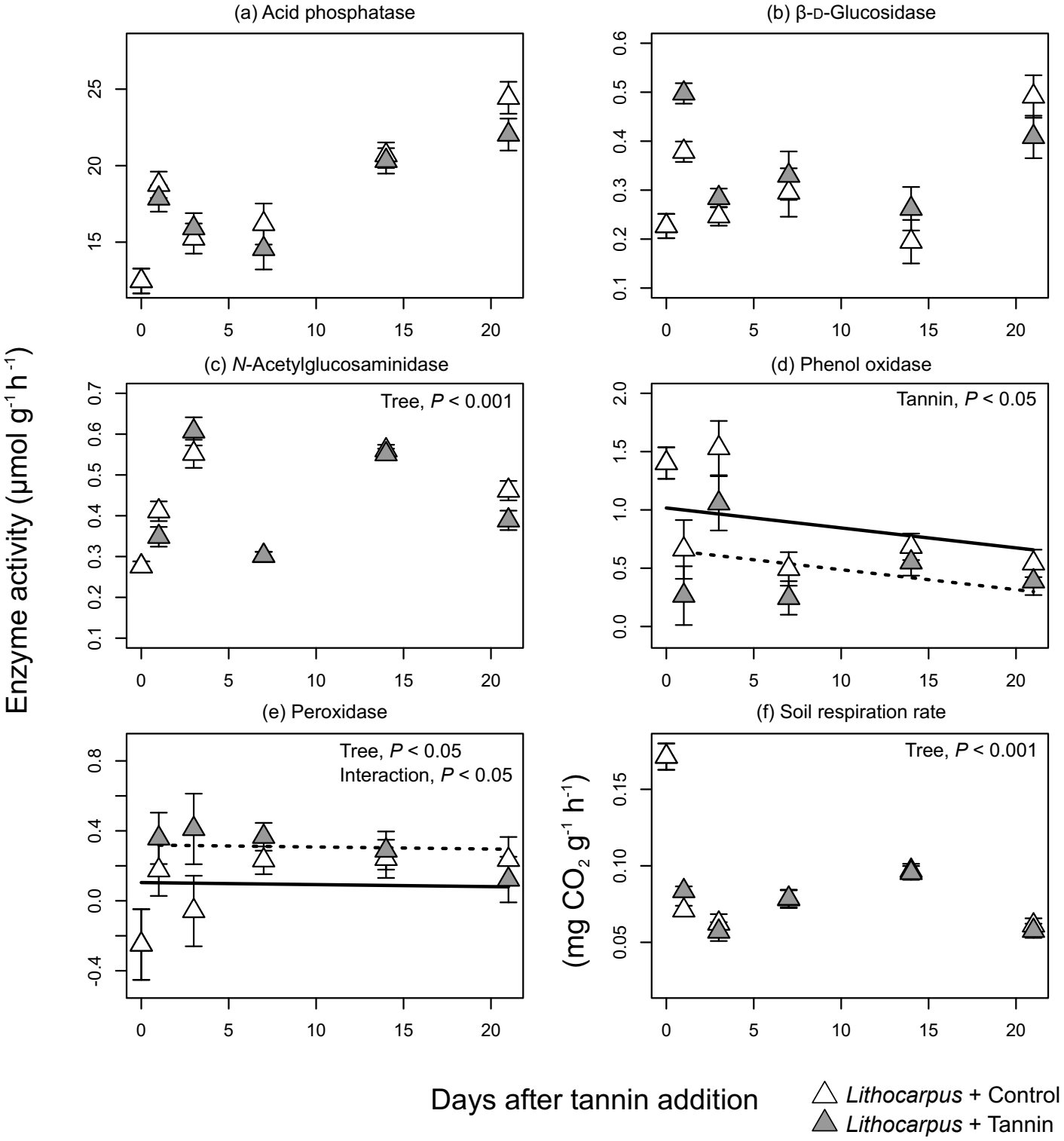

Fig. 4 Ushio et al.

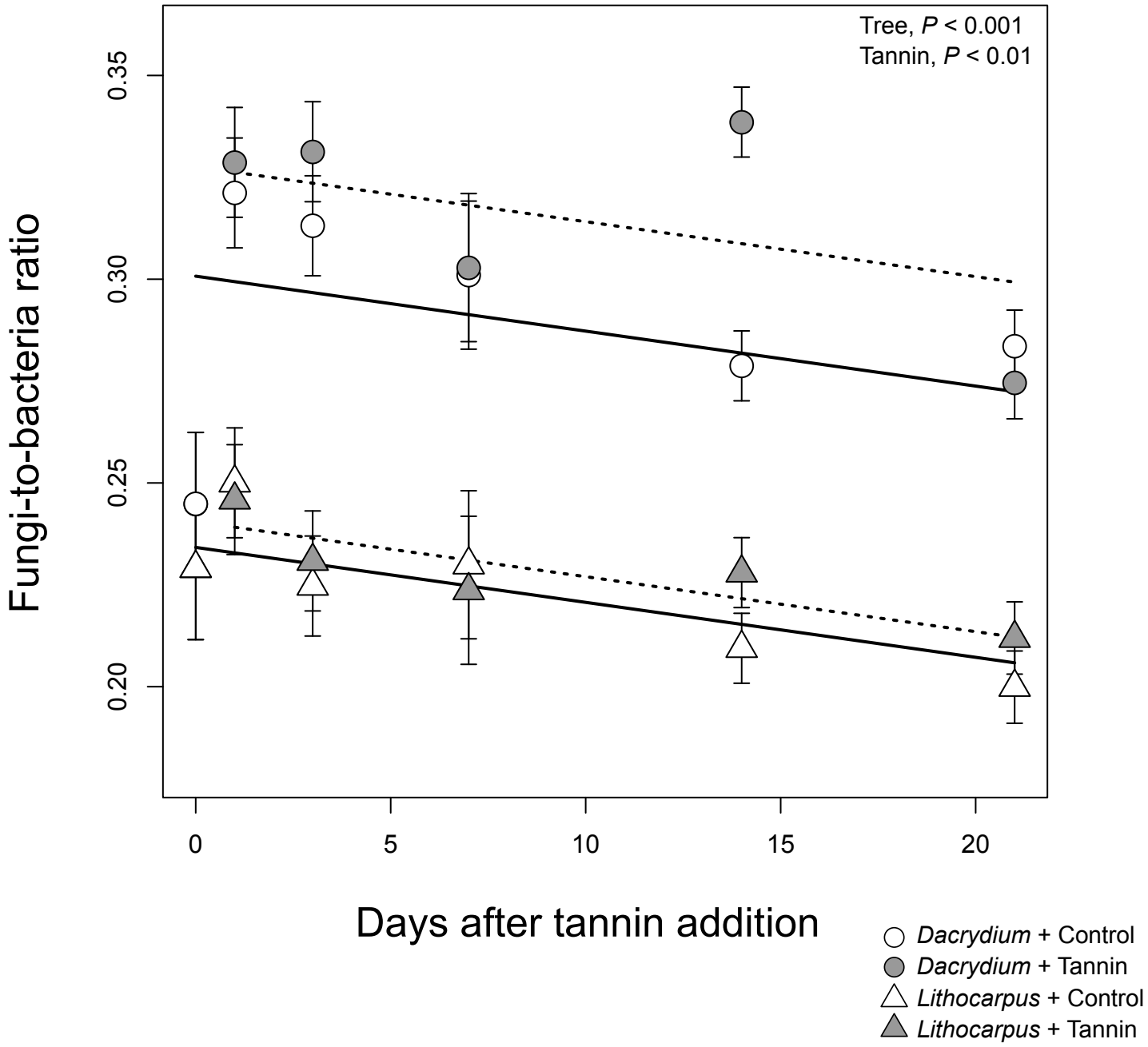

Fig. 5 Ushio et al.

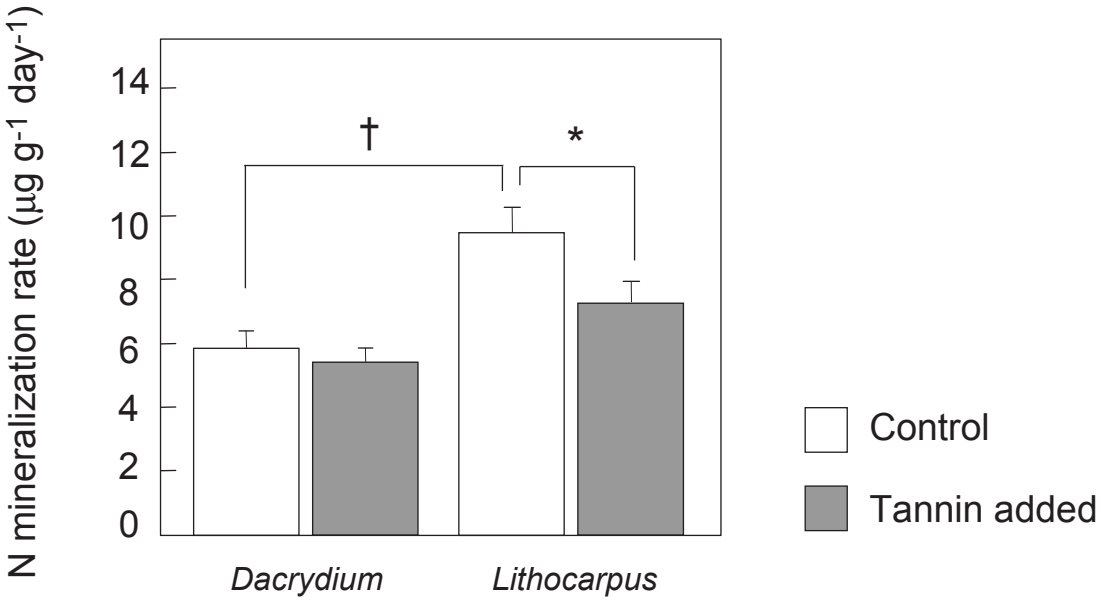

Fig. 6 Ushio et al.

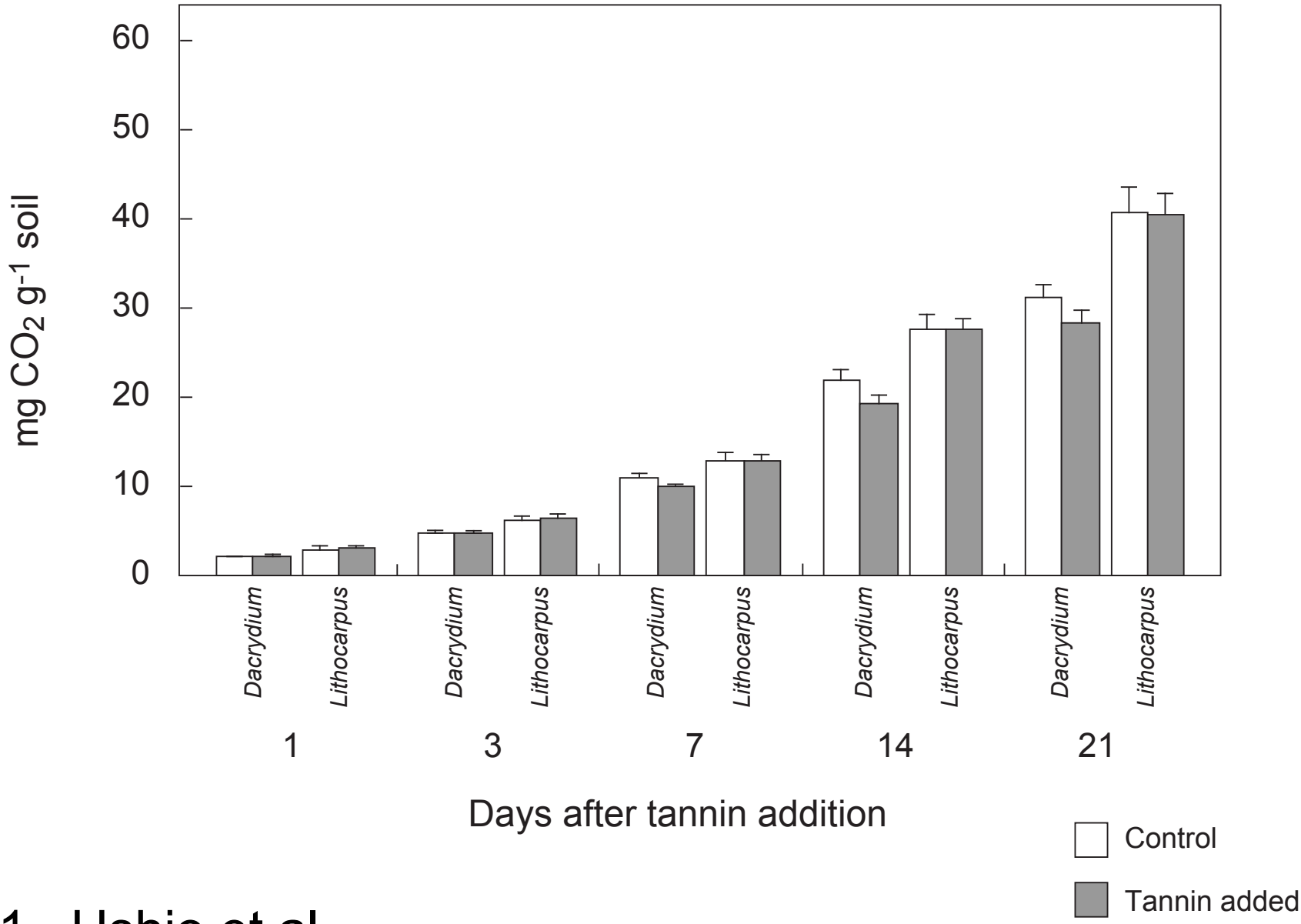

Fig. S1 Ushio et al.

701 Table 1 Coefficients and significance by linear mixed models in the tannin addition experiment under field condition

Effects	Intercept	Unit	Tree	Tannin	Interaction term
Soil microbial property					
Total lipid	1741	(nmol g^{-1})	-318	-352	233
Fungi/Bacteria ratio	0.459		**-0.174	-0.070	0.024
Gm+/Gm− ratio	2.528		0.261	0.162	†-0.307
Lipid profile PC1 (40.6%)	-0.012		0.057	-0.013	-0.041
Lipid profile PC2 (18.2%)	0.091		***-0.198	0.006	0.020
Lipid profile PC3 (10.5%)	-0.002		0.010	0.010	-0.033
Soil mineralization activity					
Acid phosphatase	29.5	(μmol g^{-1} h^{-1})	6.061	*-3.757	-1.947
β-D-Glucosidase	0.589	(μmol g^{-1} h^{-1})	0.048	*-0.061	-0.106
N-Acetylglucosaminidase	0.590	(μmol g^{-1} h^{-1})	*0.206	†-0.067	-0.090
Phenol oxidase	0.644	(μmol g^{-1} h^{-1})	†0.242	-0.335	†0.599
Peroxidase	0.752	(μmol g^{-1} h^{-1})	0.453	-0.205	-0.025
Soil respiration rate §	320	(mg CO_2 m^{-2} h^{-1})	-24.9	*-109	35.1
Soil physical property					
pH (H_2O)	4.050		*0.304	0.228	*-0.274
pH (KCl)	3.196		0.206	0.066	-0.156

702 Coefficients were estimated by the following statistical model formula: (Explained variable) = Intercept + Tree coefficient × (*Dacrydium* [0] or
703 *Lithocarpus* [1]) + Tannin coefficient × (Control [0] or Tannin [1]) + Interaction term + residuals. Therefore, intercepts indicate the mean values
704 calculated by including all treatments (i.e., two tree species and two experimental treatments). Zero and one are dummy variables. †P < 0.10, *P
705 < 0.05, **P < 0.01, ***P < 0.001. §Additive mixed model was applied only for the soil respiration rate because the respiration rate was
706 monitored periodically.

707 Table 2 Coefficients and significance by additive mixed models in the tannin addition experiment under laboratory condition

Soil properties	Intercept	Unit	Tree	Tannin	Interaction term	Days
Soil microbial property						
Total lipid	1998	(nmol g^{-1})	-234	-38.4	122	****
Fungi/Bacteria ratio	0.289		***-0.067	**0.027	-0.021	***
Gm+/Gm− ratio	1.546		0.055	†-0.063	0.035	***
Lipid profile PC1 (39.8%)	-0.100		***0.221	-0.034	0.020	
Lipid profile PC2 (21.8%)	-0.047		0.075	**0.027	-0.014	*
Lipid profile PC3 (9.5%)	-0.025		0.045	0.015	-0.021	****
Soil mineralization activity						
Acid phosphatase	21.777	(μmol g^{-1} h^{-1})	-3.365	-0.793	-0.029	****
β-D-Glucosidase	0.333	(μmol g^{-1} h^{-1})	-0.018	0.021	0.009	****
N-Acetylglucosaminidase	0.326	(μmol g^{-1} h^{-1})	***0.113	0.006	-0.022	****
Phenol oxidase	0.624	(μmol g^{-1} h^{-1})	0.249	*-0.253	-0.105	**
Peroxidase	0.474	(μmol g^{-1} h^{-1})	*-0.379	-0.065	*0.280	
Soil respiration rate	0.064	(mg CO_2 g^{-1} h^{-1})	***0.020	-0.002	-0.001	****
Cumulative CO_2	12.975	(mg CO_2 g^{-1})	**3.218	†-1.430	†1.925	****
Soil physical property						
pH (H_2O)	3.937		†0.099	**0.046	*-0.051	****
pH (KCl)	3.010		0.042	***-0.023	†0.015	****

708 Coefficients were estimated by the following statistical model formula: (Explained variable) = Intercept + Tree coefficient × (*Dacrydium* [0] or
709 *Lithocarpus* [1]) + Tannin coefficient × (Control [0] or Tannin [1]) + Interaction term + Days + residuals. Therefore, intercepts indicate the mean
710 values calculated by including all treatments (i.e., two tree species, two experimental treatments and six incubation time points). Zero and one
711 are dummy variables. †$P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

712 Table S1 Intact soil physicochemical properties and enzyme activity

Tree species		*Dacrydium gracilis* (conifer)	*Lithocarpus clementianus* (broadleaf)
Soil phycicochemical properties			
Soil Water Content	(%)	79.0 (0.36)	79.3 (1.22)
pH (H_2O)		**3.90 (0.05)***	**4.08 (0.05)**
pH (KCl)		3.08 (0.06)	3.13 (0.05)
Total phenol[1]	(μg g^{-1})	3.65 (0.39)	4.52 (0.75)
Condensed tannins[1]	(μg g^{-1})	**0.37 (0.08)** †	**0.20 (0.04)**
Soil microbial community			
Total lipid abundance[2]	(nmol g^{-1})	2309 (240)	1799 (336)
Fungi/Bacteria ratio[2]		**0.462 (0.038)****	**0.313 (0.023)**
Gm+/Gm– ratio[2]		1.552 (0.123)	1.481 (0.101)
Soil enzyme activity			
Acid phosphatase	(μmol g^{-1} h^{-1})	16.6 (0.81)	12.5 (2.18)
β-D-glucosidase	(μmol g^{-1} h^{-1})	0.174 (0.025)	0.227 (0.057)
N-Acetylglucosamidase	(μmol g^{-1} h^{-1})	**0.193 (0.012)****	**0.276 (0.016)**
Phenol oxidase	(μmol g^{-1} h^{-1})	0.750 (0.135)	1.402 (0.316)
Peroxidase	(μmol g^{-1} h^{-1})	0.625 (0.202)	N.D.
Soil C and N flux			
N mineralization rate	(μg g^{-1} d^{-1})	**5.85 (0.55)****	**9.48 (0.79)**
Nitrification rate	(μg g^{-1} d^{-1})	0.04 (0.08)	0.29 (0.21)
Soil respiration rate	(mg CO_2 g^{-1} h^{-1})	**0.125 (0.009)** †	**0.171 (0.021)**

713 [1]data of the surface organic soil beneath each tree crown, from Ushio et al. 2010. [2]data modified from
714 Ushio et al. 2008. Values of the soil enzyme activities and carbon and nitrogen flux were measured for
715 soil samples used for the laboratory incubation experiment before the incubation. Parentheses indicate
716 standard error of mean. Significant differences between the two species were indicated by †$P < 0.1$, *P
717 < 0.05 and **$P < 0.01$ (t-test).

718 Table S2 *F*-values by linear mixed models in the tannin addition experiment under field condition

Effects	Tree	Tannin	Tree × Tannin
Degree of freedom	1	1	1
Soil microbial property			
Total lipid	0.734	2.904	0.707
Fungi/Bacteria ratio	**14.78****	2.497	0.102
Gm+/Gm− ratio	0.268	0.013	**4.420**†
Lipid profile PC1 (40.6%)	0.113	1.771	0.656
Lipid profile PC2 (18.2%)	**33.36*****	1.511	0.585
Lipid profile PC3 (10.5%)	0.015	0.238	1.662
Soil mineralization activity			
Acid phosphatase	3.280	**8.194***	0.347
β-D-glucosidase	0.003	**6.389***	1.369
N-acetylglucosamidase	**9.485***	**4.403**†	0.653
Phenol oxidase	**3.721**†	0.062	**4.497**†
Peroxidase	2.358	1.325	0.004
Soil respiration rate§	0.902	**13.51***	0.715
Soil physical property			
pH (H2O)	**7.307***	3.254	**7.375***
pH (KCl)	2.101	0.069	2.909

719 Values indicate *F*-statistics. † $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. §Additive mixed model

720 was applied only for the soil respiration rate because the respiration rate was monitored periodically.

721 Table S3 *F*-values by additive mixed models in the tannin addition experiment under laboratory
722 condition

Soil properties	Tree	Tannin	Tree × Tannin	Days
Degree of freedom	1	1	1	§
Soil microbial property				
Total lipid	1.694	0.143	0.747	**14.92******
Fungi/Bacteria ratio	**20.20*****	**8.864****	2.610	**10.15*****
Gm+/Gm− ratio	0.503	**3.902†**	0.595	**13.56*****
Lipid profile PC1 (39.8%)	**59.12*****	2.448	0.431	2.527
Lipid profile PC2 (21.8%)	1.519	**8.062****	1.073	**4.672***
Lipid profile PC3 (9.5%)	2.174	1.951	1.811	**12.76******
Soil mineralization activity				
Acid phosphatase	1.505	2.599	0.002	**75.18******
β-D-glucosidase	0.127	0.762	0.076	**28.0******
N-acetylglucosamidase	**11.75*****	0.131	0.796	**61.81******
Phenol oxidase	1.695	**5.198***	0.447	**10.76****
Peroxidase	**4.140***	0.469	**4.380***	0.068
Soil respiration rate	**13.28*****	0.105	0.015	**38.25******
Cumulative CO_2	**10.78****	**3.536†**	**3.221†**	**2169******
Soil physical property				
pH (H2O)	**3.801†**	**7.098****	**4.581***	**10.02******
pH (KCl)	0.429	**13.62*****	**3.052†**	**383.3******

723 Values indicate *F*-statistics. † $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.
724 §Degree of freedom for days is not shown because additive mixed modeling does not calculate a degree
725 of freedom for a term for which the smoothing function applied.